XCV. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

PART II. THE PHOSPHORIC ESTERASE OF OSSIFYING CARTILAGE.

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In a previous communication by one of the present authors [Robison, 1923] it was shown that the bones and ossifying cartilage of young animals contain an enzyme which rapidly hydrolyses hexosemonophosphoric[1] ester and glycerophosphoric ester with liberation of free phosphate ions. The kidney was found to possess a similar hydrolytic power, but liver, spleen and pancreas were much less active in this respect, while muscle and the non-ossifying cartilage were almost or entirely without action on these monophosphoric esters.

A hexosediphosphatase, which hydrolyses at least one of the phosphoric acid groups of hexosediphosphoric ester, appeared to be more commonly distributed, being found in each of the tissues named above.

The suggestion was put forward that the former of these enzymes, the "monophosphoric esterase" present in bone, is concerned in the process of ossification by effecting the hydrolysis of phosphoric esters contained in the blood, thus bringing about a local increase in the concentration of inorganic phosphate ions, so that the product of the concentrations of phosphate and calcium ions becomes greater than the solubility product of calcium phosphate, which is thereupon deposited in the solid state.

The present paper is an account of further work on this enzyme.

Method of Extraction.

The tissues were thoroughly ground in a mortar with ten or twenty times their weight of water (or a 0·4 % solution of sodium bicarbonate) saturated with chloroform. In some of the later experiments these extracts (termed "10 %" and "5 %" respectively) were allowed to remain at 0° during 24 hours and were then filtered.

[1] Here and elsewhere in this paper "hexosemonophosphoric ester" refers to the compound isolated from the products of the fermentation of sugar by yeast juice [Robison, 1922] and not to the compound obtained by partial hydrolysis of the diphosphoric ester [Neuberg, 1918].

Such extracts prepared from bones have been kept at 0° during several months without suffering any serious diminution in their hydrolytic power, nor does there appear to be any rapid inactivation at room temperature. It is of course necessary that bacterial growth be inhibited by the addition of sufficient chloroform or other antiseptic.

Distribution of the enzyme.

In addition to the facts already reported some further information has been gained as to the occurrence of the enzyme in various animal tissues.

Bones. Active extracts have been obtained from the bones of all animals so far examined. These are: rat, rabbit, guinea-pig, kitten, chicken.

Teeth. The close similarity in the chemical composition of the inorganic portions of teeth and bones renders it very probable that the mechanism for the deposition of this material is similar in both cases. It was therefore interesting and significant to find that the phosphoric esterase is also present in teeth. Extracts prepared from the teeth of young and adult rabbits, rats and guinea-pigs usually contained the enzyme in even higher degree than those prepared from the bones of the same animals.

In one experiment "10 %" filtered extracts were prepared from the teeth and bones of young rats (three weeks old). 2 cc. of these extracts were added to 20 cc. of an $M/1000$ solution of sodium glycerophosphate and kept at 37°, the p_H being adjusted to 9·0–9·3. Colorimetric estimations of inorganic phosphate (*v.* p. 744) showed that at the end of $2\frac{1}{2}$ hours over 90 % of the glycerophosphate had been hydrolysed by both the teeth and bone extracts.

Intestines. The small intestines of rabbits, rats and guinea-pigs, washed with saline and then ground with water in the usual way, yielded extracts which hydrolysed phosphoric esters very rapidly, the activity being of the same order as that of kidney extracts. It is clear, therefore, that if phosphoric esters of the type under consideration are required by the organism they are not likely to be absorbed as such from the alimentary canal but must be formed within the body.

Variations in the activity of the tissues with the age of the animal.

Experiments were carried out with rats and rabbits of different ages, the hydrolytic power per g. of the different tissues being compared. The results were not very regular but showed in general that the superiority of the teeth and bone was most marked in very young, rapidly growing animals. In young adults the activity of the kidney was often equal to or greater than that of the bone, while in one very old rabbit the activity of the bone and teeth fell far below that of kidney and intestine and even of the liver. This falling off in the activity may be explained partly by the increased proportion of inorganic matter relative to the growing surfaces of the bone as well as by the reduction in the activity of the enzyme-secreting cells.

The optimum hydrogen ion concentration.

The exact determination by electrometric methods of the optimum hydrogen ion concentration has not yet been carried out, but sufficient information for our immediate purpose was furnished by the following experiment:

A number of tubes was prepared, each containing:
1 cc. { of a solution ($\overline{HP}_1$) of potassium hexosemonophosphate (containing 2·64 mg. P),
{ or a solution ($\overline{GP}_1$) of potassium glycerophosphate (containing 2·66 mg. P).
0·5 cc. of a 2½ % extract of epiphyseal cartilage of young rats (macerated but not filtered).
0·02 cc. chloroform.
0·1 cc. of a ·02 % solution of phenol red or thymol blue.
0·3 cc. *N*/50 NaOH or *N*/50 H_2SO_4 or H_2O as required to adjust the p_H.

The reaction was adjusted so that the tubes formed a series of varying p_H from 6·8–9·9. They were kept at 37° during 18 hours and the inorganic phosphate in each tube was then estimated by the modified Neumann method previously described [Robison, 1923]. Controls containing the solutions of phosphoric esters without cartilage extract and the latter without ester were treated in the same way. The very small amounts of inorganic phosphate found in these controls were subtracted from those found in the other tubes in order to determine the amount of hydrolysis effected by the enzyme.

Table I.

p_H before incubation	p_H after incubation	Inorganic phosphate (mg. P) less that in controls		% of phosphoric ester hydrolysed	
		$\overline{HP}_1$	$\overline{GP}_1$	$\overline{HP}_1$	$\overline{GP}_1$
Phenol red series:					
6·8	7·0	0·12	0·15	5	6
7·0	7·4	0·21	0·20	8	8
7·4	7·8	0·275	0·355	10	13
7·6	7·9	0·36	0·47	14	18
8·0	8·2	0·75	0·80	28	30
8·4	8·4	1·515	1·52	57	57
Thymol blue series:					
8·4	—	1·37	1·51	52	57
9·0	—	1·42	1·48	54	56
9·4	—	1·51	1·49	57	56
9·9*	—	0·82	0·21	31	8

* This value, being near the extreme limit of the colour range of thymol blue, was somewhat uncertain.

The activity of the enzyme is thus greatest in markedly alkaline solutions, the optimum p_H being 8·4–9·4 (determined colorimetrically). Between these limits the activity is practically constant, but on either side of them it falls off rapidly, the rate of hydrolysis at the normal p_H of the blood being only about one-fifth of that at the optimum.

At the same hydrogen ion concentration the rates of hydrolysis of hexosemonophosphoric ester and glycerophosphoric ester are the same.

In the experiments previously described the solutions of the esters were made just neutral to phenolphthalein before the addition of the tissue extracts, so that the final p_H was a little below the optimum in the region where a slight change in reaction makes considerable difference in the rate of hydrolysis.

We have therefore repeated some of these comparative tests at a p_H lying well within the limits for optimum activity of the enzyme. The results show the same marked superiority in the hydrolytic power of the bone extracts over those of the other tissues with the exception of the kidney and intestine. The difference between bone on the one hand and liver and pancreas on the other is even more pronounced at the higher p_H, which points to the probability that different enzymes are concerned.

In one experiment the tissues of two rats, 27 days old, were separately macerated with twenty times their weight of a 0·4 % solution of sodium bicarbonate, saturated with chloroform. These "5 %" extracts were used without filtration. A portion of the bone extract was diluted with an equal volume of sodium bicarbonate solution, thus making a "2½ %" extract.

Tubes were prepared containing

1 cc. { water, or solution ($\overline{HP_1}$) of potassium hexosemonophosphate (containing 2·64 mg. P), or solution ($\overline{GP_1}$) of potassium glycerophosphate (containing 2·66 mg. P).
0·5 cc. tissue extract.
0·1 cc. 0·4 % solution of thymol blue.
0·02 cc. chloroform.
0·1 cc. *N*/10 NaOH solution or water.

The p_H was adjusted to 9·0–9·2 and the tubes were kept during 18 hours at 37°, after which the inorganic phosphate was estimated by the modified Neumann method. The results are shown in Table II.

Table II.

Tissue	Extract %	Inorganic PO_4 (mg. P) after 18 hrs. at 37° less that in controls			% of phosphoric ester hydrolysed	
		H_2O	$\overline{HP_1}$	$\overline{GP_1}$	$\overline{HP_1}$	$\overline{GP_1}$
Bone cartilage (epiphyses)	5	·03	2·44	—	92	—
" "	2½	·02	2·06	2·25	78	85
Rib cartilage (unossified)	5	·06	0·04	—	2	—
Kidney	"	·09	1·87	2·20	71	83
Liver	"	·03	0·27	0·22	10	8
Spleen	"	·03	0·46	0·51	17	20
Pancreas	"	·06	0·03	0·08	1	3
Muscle	"	·09	—	0·25	—	9

Proportionality between amount of enzyme and change effected does not of course exist when the latter amounts to 70–80 % of the total substrate, but it is clear that in the above experiment the activity of the epiphyseal cartilage relative to that of the kidney was about 2 : 1 and to that of the spleen, liver and pancreas decidedly greater than 8 : 1, 16 : 1 and 80 : 1 respectively.

The question whether the hydrolytic power of the different tissues with respect to the monophosphoric esters is due in each case to the same enzyme was further investigated by comparing the relative activities at different H^+ concentrations. The tests were carried out with "5 %" filtered extracts prepared from tissues of several rats of the same litter (10 weeks old), sodium glycerophosphate being the substrate. Four series of tubes were prepared;

in one the p_H was adjusted to 7·3 (indicator, phenol red), in the second to 8·3 (indicator, phenolphthalein), and in the third to 9·3 (indicator, thymolphthalein). The fourth series contained water in place of glycerophosphate and served as controls. The extracts of bone, teeth and kidney were used in a dilution of 1 in 5 (*i.e.* "1 %" extract) for the series at p_H 8·3 and p_H 9·3. The amounts of ester hydrolysed were thus rendered more comparable, in no case exceeding 40 % of the total substrate.

The inorganic phosphate set free in 18 hours at 37° was, in this experiment, estimated colorimetrically by the Briggs modification of the Bell-Doisy method, proteins being first precipitated by trichloroacetic acid. The ratios of the amounts of hydrolysis effected by each tissue at p_H 9·3 and 8·3 to that effected at p_H 7·3, as shown in Table III, confirm the supposition that the activity of the liver and pancreas and probably also of the spleen is due to a different enzyme from that in the bone and kidney.

Table III.

Tissue	Ratio of amounts of ester hydrolysed at p_H 7·3	8·3	9·3
Bone	1 :	3·5 :	5·5
Teeth	1 :	3·7 :	5·8
Kidney	1 :	3·0 :	5·1
Liver	1 :	0·6 :	1·0
Spleen	1 :	2·0 :	3·2
Pancreas	1 :	0·2 (?) :	1·5

Action of the bone enzyme on olive oil and other esters.

The marked difference between the bone enzyme and the pancreatic lipase and esterase was again brought out in the comparison of their actions on olive oil and other esters. The experiment was carried out at the same time as the preceding one and with the same 5 % tissue extracts.

The following solutions were used for the substrates:

(*a*) Olive oil emulsion containing 0·2 % bile salt.

(*b*) Triacetin solution (1 % by volume).

(*c*) Ethyl butyrate solution (1 % by volume).

5 cc. of the solution were added to 5 cc. of the 5 % tissue extract (bone or pancreas) or to the same volume of extract which had previously been heated to 100°. 0·5 cc. phenolphthalein solution (·1 %) was added to each tube of the series containing olive oil and 0·5 cc. phenol red solution (·02 %) to each of the others. The p_H was adjusted to 8·4 in each case and the tubes were placed in a thermostat at 37°. At short intervals (five minutes at first) the contents of the tubes were titrated to p_H 8·4 with 0·1 *N* sodium hydroxide solution and the total amounts thus added during 40 minutes and 3½ hours are shown in Table IV. At the end of 3½ hours alcohol was added to the tubes containing olive oil emulsion and the titration of the free acid carried out in the usual way. The amounts of sodium glycerophosphate hydrolysed

in 18 hours by 1 cc. extract are also shown for comparison with the amounts of the esters hydrolysed in 3½ hours by 5 cc.

Table IV.

0·1 *N* sodium hydroxide.

		Bone extract	Bone extract heated to 100°	Difference = amount hydrolysed	Pancreas extract	Pancreas extract heated to 100°	Difference = amount hydrolysed
Olive oil	40 mins.	—	—	—	—	—	—
	3½ hrs.	0·26	0·32	−0·06	22·1	0·33	21·77
Triacetin	40 mins.	0·33	0·07	0·26	1·46	0·07	1·39
	3½ hrs.	1·12	0·46	0·66	2·98	0·46	2·52
Ethyl butyrate	40 mins.	0·20	—	0·20	1·58	—	1·58
	3½ hrs.	1·06	—	1·06	1·97	0·07	1·90
Sodium glycerophosphate (+1 cc. bone extract, 18 hrs. at 37°)		—	—	2·56 mg. P = 0·82 cc. 0·1 *M* soln.	—	—	0·029 mg. P = 0·009 cc. 0·1 *M* soln.

The rate of hydrolysis of ethyl butyrate by the pancreas had fallen to zero before the end of 3½ hours, while that of triacetin had become very slow. This was probably owing to the effect of the reverse reaction since the hydrolysis was by no means complete. The figures for 40 minutes give therefore a fairer basis for comparison.

The bone extract thus hydrolysed ethyl butyrate, triacetin and glycerophosphoric ester at about the same rate, but had no action on olive oil. The pancreas extract, which hydrolysed the oil very rapidly, was also six or seven times as active as the bone extract towards ethyl butyrate and triacetin. Towards the phosphoric ester, however, its activity was less than 1/80 of that possessed by the bone extract.

Action of the bone enzyme on lecithin.

The proof that glycerophosphoric ester and hexosemonophosphoric ester are hydrolysed with equal rapidity by the bone enzyme, together with the fact that lecithin, which is a derivative of glycerophosphoric ester, is known to be present in the blood, while the presence of hexosemonophosphoric ester is still unproven, made it desirable to examine the action of the enzyme on lecithin.

A quantity of this substance was prepared from egg yolk and was used in the crude condition without removal of the kephalin. It was made up into an emulsion of about *M*/100 concentration with a 0·1 % solution of bile salt. Three flasks were made up as follows:

LB. 25 cc. *M*/100 lecithin emulsion + 5 cc. 5 % bone extract.
LO. 25 cc. *M*/100 lecithin emulsion + 5 cc. 5 % bone extract heated to 100°.
GB. 25 cc. *M*/100 sodium glycerophosphate solution containing 0·1 % bile salt + 5 cc. 5 % bone extract.

One drop of phenolphthalein was added to each flask and the reaction was adjusted till a faint pink tint was visible (p_H 8·3–8·4). 5 cc. of the liquid were at once removed from each flask and run into 1 cc. of 15% trichloroacetic acid solution. After shaking and filtering, a measured quantity of the

protein-free filtrate was taken for the estimation of inorganic phosphate by Briggs' method. The flasks were placed in a thermostat at 37° and the inorganic phosphate again estimated at intervals. The results are shown in Table V. In another experiment a solution similar to "LB" given above, but with phenol red in place of phenolphthalein, was kept for six hours at 37° without any change in the p_H being observed. It would seem therefore that neither the fatty acids nor phosphoric acid are set free from lecithin by the bone enzyme.

Table V.

	Inorganic PO_4 (mg. P) in 5 cc.		
Time at 37° (hrs.)	LO	LB	GB
0	None	0·035	0·042
$2\frac{3}{4}$	,,	—	0·252
$22\frac{1}{2}$	,,	0·033	0·702
Amount hydrolysed	—	None	0·660
	Total P (mg. P) in 5 cc.		
	1·46	—	1·32

DEPOSITION OF CALCIUM PHOSPHATE IN BONES OF RACHITIC ANIMALS.

We have continued the experiments on the deposition of calcium phosphate in the bones of rachitic animals of which brief mention was made in the previous paper [Robison, 1923]. It was there shown that the bones of rachitic rats contained the enzyme in a very high degree, the extracts being relatively more active than those of normal animals. This suggested the possibility that the immediate cause of the failure to deposit calcium phosphate in these bones might be the lack of the necessary substrate, some phosphoric ester, in the blood. If this were so, these poorly calcified bones, if immersed under suitable conditions in a solution of calcium hexosemonophosphate or calcium glycerophosphate might, by hydrolysis of the ester, show a deposit of calcium phosphate in those portions where normally calcification should have taken place. A considerable measure of success was realised at the first attempt, but a large number of trials had to be made before the best conditions were discovered. The chief difficulty lay in the fact that diffusion of the substrate into the bone is a very slow process compared with the constant supply of material by the blood stream to those points where it is required. Probably for this cause we were not successful in our attempts to carry out the experiment with solutions containing approximately the same amounts of calcium and total phosphorus as are found in the blood. By using stronger solutions of the ester and immersing the bones for a sufficient length of time, a considerable deposit of calcium phosphate was obtained, but this was limited by the simultaneous diffusion of the enzyme causing a general deposit of calcium phosphate in the tube and throughout the bone. In all our later experiments the p_H of the solution has been adjusted to lie within the optimum range for the enzyme. *i.e.* 8·4–9·4. Whether there is in ossifying cartilage some mechanism for raising the p_H of the plasma bathing the cells is a question that cannot at present be answered, but *in vitro* the deposition

of calcium phosphate certainly proceeds more rapidly under such conditions. Calcium hexosemonophosphate and calcium glycerophosphate were used as substrates with equal success.

Method of experiment. The bones and ribs used in these experiments were taken from young rats that had been fed during three to four weeks on a diet deficient both in the fat-soluble *A* factor and in phosphorus (McCollum diet No. 3143). They were removed and immersed in the solution as rapidly as possible after the animals were killed. In some cases the bones were used intact, while in others they were cut in half longitudinally, one half being used as a control. After immersion in the solutions of calcium hexosemonophosphate or calcium glycerophosphate at 37° for the specified time, they were rinsed several times with saline, fixed in formalin, dehydrated and embedded in the usual way. It was of course necessary that the sections should be cut without preliminary decalcification. Staining was carried out by immersing the section for 30 minutes in a 1 % solution of silver nitrate during exposure to bright light (von Kossar's method).

We take it that the staining of the calcified portion of bone by this method is to be explained thus. The silver nitrate reacts with the calcium phosphate which is very slightly soluble in water forming the still less soluble yellow silver phosphate which is then reduced to metallic silver by the action of the light, accelerated by the organic matter of the bone. If any soluble phosphates, chlorides or other salts, which form insoluble silver compounds, were present, these also would yield a black stain, but such compounds should be entirely removed in the preliminary treatment. We were, however, anxious to make certain that the new deposit observed in our experiments was really derived from calcium phosphate set free by the action of the enzyme on the phosphoric ester, and not to the presence of calcium glycerophosphate itself or any other salt retained by the cartilage merely by adsorption or in some form of physical or chemical combination. We therefore carried out a series of controls by immersing the other halves of the same bones, or similar bones from the same animals, in solutions of sodium chloride, sodium phosphate and calcium chloride. The results uniformly obtained after satisfactory conditions had been determined leave no doubt that fresh calcium phosphate had been deposited by the action of the enzyme on the phosphoric ester in those bones treated with the solutions of calcium hexosemonophosphate and calcium glycerophosphate. The controls were in all cases negative. The difference in the two groups was indeed at once apparent to the naked eye when the sections were stained. On microscopic examination it was seen that in the controls the broad zone of hypertrophic cartilage, characteristic of rickets, remained entirely uncalcified. In the sections of the bones that had been immersed in the solutions of the phosphoric esters this hypertrophic zone was deeply stained, and under higher magnification this was seen to be due to the presence of minute granules thickly deposited throughout the matrix of the cells. The deposit was most dense in the zone of provisional calcification, only a few

discrete particles being seen among the cells of the proliferating cartilage, and none at all in the resting cartilage.

In these bones the periosteum was also deeply stained, which led us to carry out a similar experiment with periosteum stripped from the bone of a rachitic rat before immersion in the solutions. After treatment with calcium glycerophosphate the sections showed a deep brown staining extending from the inner surface through about one-third of the tissue, while in the control no staining at all occurred.

The photomicrographs shown in Plates IV and V illustrate the results of these experiments.

Fig. 1, Plate IV. Section of half a humerus from a severely rachitic rat, after being soaked in disodium phosphate solution at 37° for eight hours (p_H 8·6–8·8).
RC = Resting cartilage.
PC = Zone of proliferating cartilage.
HC = Zone of hypertrophic cartilage, which is entirely uncalcified.
M = Metaphisis.
Co = Cortex.
Stained with silver nitrate and counterstained with eosin.
Magnification × 15.

Fig. 2, Plate IV. Section of the other half of the same humerus as in Fig. 1, but soaked in a solution of calcium hexosemonophosphate at 37° for eight hours (p_H 8·6–8·8). The entire zones of proliferating and hypertrophic cartilage are here deeply stained. There is a dense precipitation of granules of calcium phosphate in the matrix of the cells. With a higher magnification granules can also be seen scattered lightly over the surface of the cells themselves. The general appearance seems to indicate that considerable diffusion of the enzyme has taken place during the lengthy immersion.
Stained with silver nitrate and counterstained with eosin.
Magnification × 15.

Fig. 3, Plate IV. Section of rib of slightly rachitic rat, immersed intact in a solution of disodium phosphate for 12 hours at 37° (p_H 8·4). There is no fresh calcium phosphate deposition in either the periosteum or the zone of hypertrophic cartilage.
Stained with silver nitrate; no counterstain.
Magnification × 30.
Sections of other control ribs from the same rat soaked in (1) sodium chloride, (2) calcium chloride present the same appearance.

Fig. 4, Plate IV. Rib from the same rat as in Fig. 3. Immersed intact in calcium glycerophosphate solution for 12 hours at 37° (p_H 8·4). The zone of hypertrophic cartilage and the periosteum are stained dark brown. Some deposit is also to be seen in the lower part of the zone of proliferating cartilage.
Stained with silver nitrate; no counterstain.
Magnification × 30.
Although the period of immersion was somewhat longer than with the divided bone shown in Figs. 1 and 2, Plate IV, diffusion would not seem to have occurred to the same extent in these intact bones.

Fig. 5, Plate V. A drawing made from the portion of Fig. 3 (Plate IV) enclosed in the rectangle.
Magnification × 320.
No granules are to be seen.

Fig. 6, Plate V. Drawing made from the part of the rib in Fig. 4 (Plate IV) enclosed in the rectangle, showing the relative size and density of the calcium phosphate granules deposited in the different zones of the cartilage.
Magnification × 320.

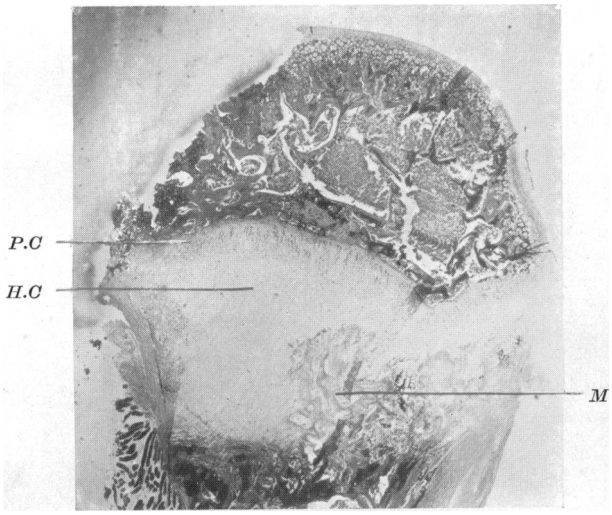

Fig. 1.

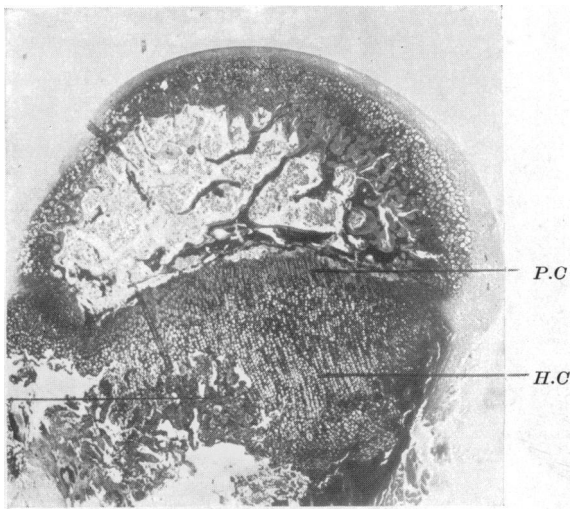

Fig. 2.

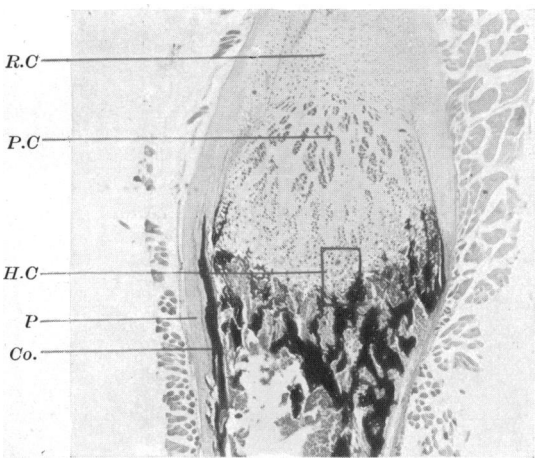

Fig. 3.

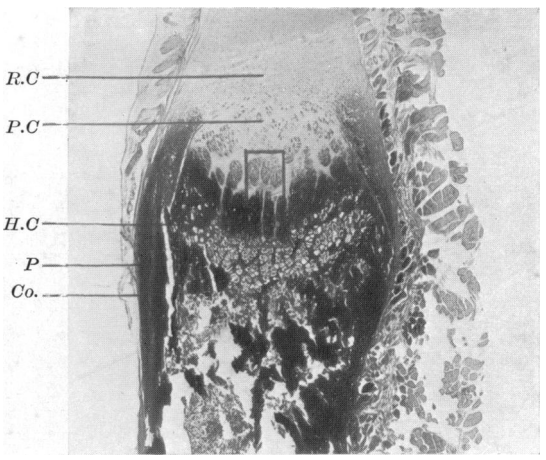

Fig. 4.

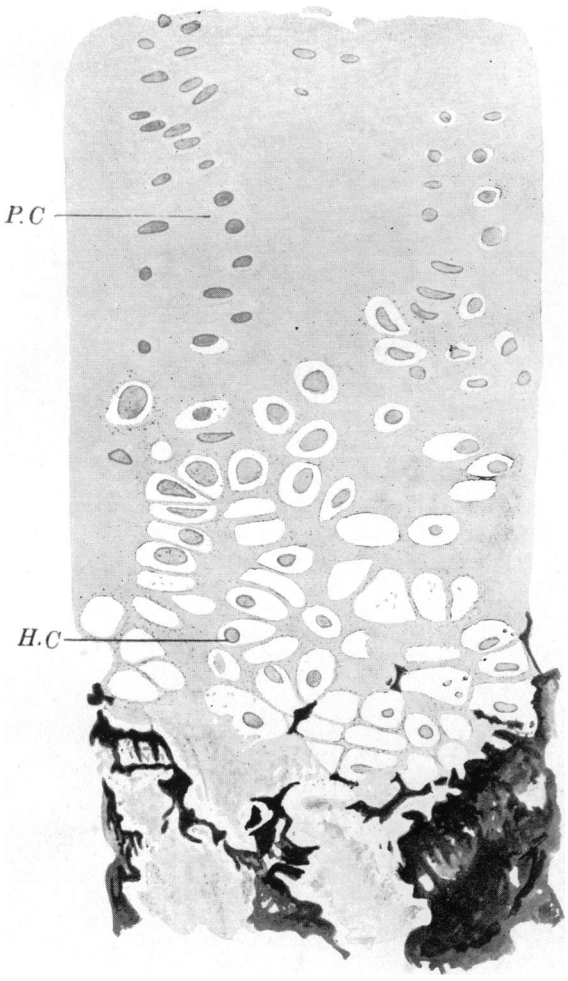

Fig. 5.

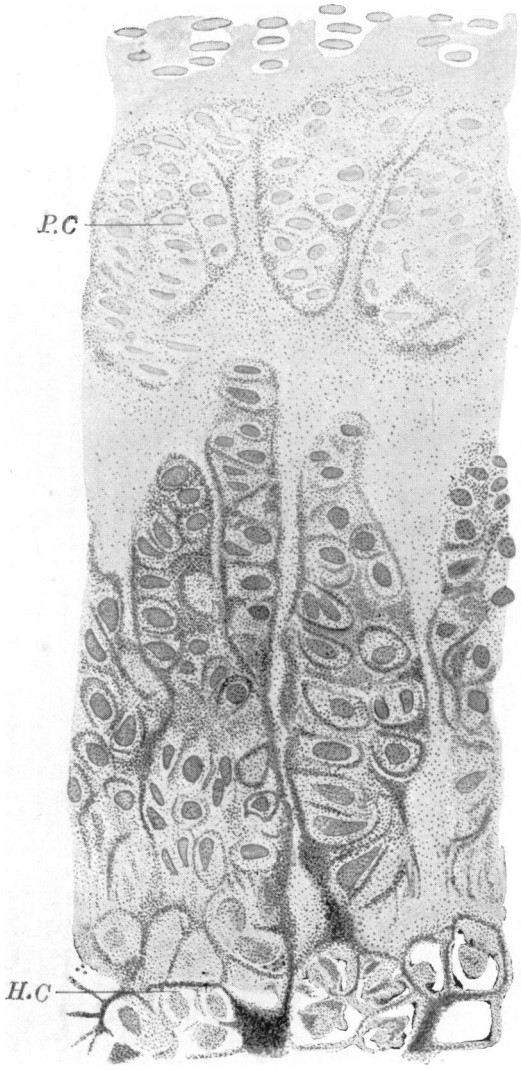

Fig. 6.

Discussion.

The simplest way of considering the relationship between the inorganic portion of bone and the fluid by which it is bathed, is as that of a solid in contact with its saturated solution. If for the moment we assume that the solid is tricalcium phosphate, the reactions taking place are

$$Ca^{++} + PO_4^{\equiv} \longleftrightarrow \underset{\text{dissolved un-ionised}}{Ca_3(PO_4)_2} \longleftrightarrow \underset{\text{solid}}{Ca_3(PO_4)_2},$$

and the equation

$$[Ca^{++}] \times [PO_4^{\equiv}] = K\,[Ca_3(PO_4)_2] = k \text{ (solubility product)}$$

should hold for this system as for a saturated solution of the salt in pure water, except that the value of k will not necessarily be the same but may be modified by the other substances dissolved in the tissue fluid. If the concentrations of the Ca and PO_4 ions are such that the product is greater than k more solid calcium phosphate will be deposited, whereas if the product is less than k some of the solid will be re-dissolved. Both deposition and re-solution occur in the body, the former during normal bone formation, the latter under special conditions such as that of pregnancy if the increased demand of the organism for calcium is not satisfied by the diet. The equilibrium is conditioned by the concentration of the above ions and not directly by that of the total calcium and phosphate present. According to Rona and Takahashi [1913] and Clark [1923], a portion of the calcium of serum is present in non-diffusible form, probably in combination with protein. This still leaves the concentration of diffusible calcium in the serum greatly in excess of that corresponding with the solubility of $CaCO_3$, under similar conditions with regard to the HCO_3 content and p_H, as determined by Rona and Takahashi [1913], from which they concluded that serum forms a metastable supersaturated solution of calcium bicarbonate (? carbonate). Their solubility figure, 22 mg. Ca per litre at 18°, has been confirmed by Brinkman and van Dam [1920] who also devised a method by which the calcium ions alone could be estimated. Measured amounts of oxalate solution were added until the product of calcium and oxalate ions exceeded the solubility product of calcium oxalate, which was precipitated, giving rise to a turbidity. In this way they determined the ionised calcium in serum and found amounts corresponding closely with the solubility figure of 22 mg. per litre.

The whole of the inorganic phosphate in serum was found by Rona and Takahashi [1913] to be present in diffusible form, but of this phosphate only a very small proportion will be present as $[PO_4^{\equiv}]$ at the p_H of the blood.

To consider the plasma as supersaturated with respect to calcium carbonate and phosphate does not seem justified until more is known of the factors influencing the degree of ionisation and the solubility product. A supersaturated solution might, it is true, deposit calcium salts only on coming into contact with the solid phase, *i.e.* bone, but this would not explain the

beginning of ossification in embryonic cartilage, nor the ease with which the organism draws on its store of calcium salts in the skeleton when it has more urgent need of them for other purposes.

If then the plasma or tissue fluid be considered as a saturated solution of these salts, we must look for some factor by which a localised increase in the concentration of one or both ions may be brought about. With the interesting possibility of an increase in the proportion of ionised calcium we are not for the present concerned. Experiments on this point have been reported by Clark [1923].

The concentration of $PO_4^{\equiv}$ ions might be increased in two ways: 1) by an increase of total inorganic phosphate, (2) by a shift of p_H towards the alkaline side, thereby increasing the $PO_4^{\equiv}$ at the expense of HPO_4^{-} ions. As we have shown, the bone enzyme supplies one factor by which, in presence of a suitable phosphoric ester, the concentration of inorganic phosphate and therefore of $PO_4^{\equiv}$ ions could be increased. The experiments described in this paper confirm the previous suggestion [Robison, 1923] that this enzyme is secreted in an active condition in those parts of the bone, and only those parts, where ossification normally takes place.

The demonstration of calcium phosphate deposition, *in vitro*, in bones taken from rachitic animals and immersed in solutions of suitable phosphoric esters, not only strengthens the probability that the enzyme is actively concerned in ossification, but also furnishes information with regard to the areas of the bone in which it is secreted. The probability that some diffusion of the enzyme takes place after removal of the bone and during the experiment must, however, be reckoned with, and comparison of the stained sections obtained after immersion of the bones for different lengths of time leads to the conclusion that in those experiments which yielded the most deeply stained sections (Fig. 2, Plate IV) such diffusion had occurred.

To what extent, then, can the stained areas in sections obtained in the less prolonged experiments such as those shown in Figs. 4 and 6, Plates IV and V, be taken as indicating the normal location of the enzyme? By examining the apparently unstained sections obtained in the shortest experiments (the bones immersed for two hours only) under high magnification it was seen that a slight sprinkling of fine particles was present in the same areas in which a more obvious deposit was found, in bones from the same rat after eight hours' immersion.

Without wishing to make too definite an assertion, we think that this indicates the probability that the enzyme had actually been secreted throughout this area during life, and had not merely found its way by diffusion from some other part of the bone after its removal from the animal.

The fact that the enzyme is most active at a p_H between 8·4 and 9·4 suggests a further interesting possibility, that osteoblasts or hypertrophic cartilage cells have the power to raise the p_H of the tissue fluids. Such a change of reaction would at once increase the concentration of $PO_4^{\equiv}$ ions by shifting

the equilibrium between them and the $HPO_4^{=}$ and would at the same time increase the activity of the enzyme. It would also shift the equilibrium between $HCO_3^{-} \longleftrightarrow CO_3^{=}$ further to the right and thus cause a deposit of calcium carbonate which might conceivably account for the presence of this substance to the extent of about 10 % in the inorganic portion of bone.

We have attempted to obtain proof of such an alkaline reaction in the ossifying cartilage by intra-vital injections of thymol blue, phenol red and other indicators. Rats tolerated a series of such injections into the heart but though, on removal of the bones immediately after death, we found the surrounding muscles stained, we have not yet succeeded in obtaining penetration of the stain into the cartilage. Macerated extracts of bones, after standing at 37°, frequently had a p_H of about 8·4, but this might be due to re-solution of the calcium salts from the solid bone. These experiments are being continued.

As regards the chemical composition of the inorganic deposit in bone and teeth, it has been suggested by Gabriel [1894] and others that the chief constituent is a combined carbonate-phosphate of calcium. Some of Gabriel's analyses are shown below and illustrate the very close agreement in chemical composition of these deposits.

Percent. of	Human bone	Ox bone	Ox teeth
CaO	51·31	51·28	50·76
MgO	0·77	1·05	1·52
K_2O	0·32	0·18	0·20
Na_2O	1·04	1·09	1·16
Water of crystallisation	2·46	2·33	2·21
P_2O_5	36·65	37·46	38·88
CO_2	5·86	5·06	4·09
Cl	0·01	0·04	0·05
Water of constitution...	1·32	1·37	1·27
Total...	99·74	99·86	100·14

Bassett, Jr. [1917] has shown that the solid phase in equilibrium with solutions containing calcium and phosphate under the conditions found in normal serum is hydroxyapatite $(Ca_3P_2O_8)_3Ca(OH)_2$, a compound even more basic than tertiary calcium phosphate. He considers that the mineral constituents of bone consist in the main of this compound mixed with a certain amount of calcium carbonate. The traces of magnesium, sodium and potassium and of chloride he considers to be carried down by adsorption. The solubility relationships of the various calcium phosphates have not yet been satisfactorily determined, but it is somewhat surprising to find suggested in several recent papers the idea that the solubility product of $CaHPO_4$ is the limiting factor in the deposition of bone. This conception is developed at some length by Howland and Kramer [1923] who have attempted to show that rickets is associated with a low product of calcium and phosphate concentrations in the blood.

They recognise that calcium exists in the bone as tertiary calcium phosphate, but do not understand how this can be formed, and state that "While

the transformation from the secondary to the tertiary phosphate can be brought about with ease in the test-tube by lowering the carbon dioxide tension it does not appear likely that such a profound alteration could take place in the body (change of reaction to p_H 12) as is required to bring about a precipitation of tertiary calcium phosphate."

In this argument they forget that, although at p_H 7·4 the concentration of $PO_4^{\equiv}$ may be extremely small in relation to that of $HPO_4^{=}$, it is nevertheless not "insignificantly small," but is the determining factor as regards deposition of the solid phase owing to the very slight solubility of the tertiary salt.

In another paper by Kugelmass and Shohl [1924] there appears the statement: "At the hydrion concentration of blood $CO_3^{=}$ and $PO_4^{\equiv}$ are negligible and so the equilibria involve only the ions Ca^{++}, H^+, HCO_3^-, $HPO_4^{=}$ and $H_2PO_4^-$ and the molecules CO_2, H_2CO_3, $Ca(HCO_3)_2$, $Ca(HPO_4)$ and $Ca(H_2PO_4)_2$."[1]

The equilibria between un-ionised, dissolved $Ca_3(PO_4)_2$ and $CaHPO_4$ and the ions derived from them are expressed by the following equations:

$$(1)\quad [Ca^{++}] \times [PO_4^{\equiv}] = K_1 [Ca_3(PO_4)_2].$$

$$(2)\quad [Ca^{++}] \times [HPO_4^{=}] = K_2 [CaHPO_4].$$

$$(3)\quad [H^+] \times [PO_4^{\equiv}] = K_3 [HPO_4^{=}].$$

If k_1 and k_2 are the solubility products of $Ca_3(PO_4)_2$ and $CaHPO_4$ respectively, then if the concentrations of calcium and total inorganic phosphate are sufficiently increased, deposition of solid salt will occur (if supersaturation be excluded) when either

$$[Ca^{++}] \times [PO_4^{\equiv}] > k_1 \text{ the solid being in this case } Ca_3(PO_4)_2$$

or $$[Ca^{++}] \times [HPO_4^{=}] > k_2 \quad ,, \quad ,, \quad CaHPO_4.$$

Which of these conditions is realised depends on the values of k_1, k_2, K_3 and $[H^+]$.

The solution will come into equilibrium with solid $Ca_3(PO_4)_2$ or will deposit this salt if $\frac{[PO_4^{\equiv}]}{k_1} > \frac{[HPO_4^{=}]}{k_2}$, or, since $[PO_4^{\equiv}] = \frac{K_3 [HPO_4^{=}]}{[H^+]}$, if $\frac{K_3}{k_1 [H^+]} > \frac{1}{k_2}$, *i.e.* if $[H^+] < K_3 \frac{k_2}{k_1}$.

There will therefore be a limiting value for $[H^+]$ below which $Ca_3(PO_4)_2$ is deposited and above which $CaHPO_4$ is deposited. This value depends on the constants k_1 k_2 K_3 and need not correspond with a p_H of 12 as suggested by Howland and Kramer. That the limiting p_H is much lower than that of normal blood has long been known but was demonstrated very simply

[1] In the same paper these authors make use of Walker and Cormack's [1900] value for the first dissociation constant of H_2CO_3 ($3{\cdot}04 \times 10^{-7}$) and state that their own experiments confirm Walker and Cormack's conclusion that the fraction of total CO_2 existing in solution as H_2CO_3 is greater than 0·5. They ignore the work of Thiel and Strohecker [1914] who have shown that in a 0·00812 *M* solution of carbon dioxide only 0·67 % of the total CO_2 is present as carbonic acid, and that the dissociation constant of this acid is 5×10^{-4}, that is, it is twice as strong as formic acid.

by the following experiment. 10 cc. of a solution containing 10 mg. of phosphorus in the form of KH_2PO_4 and 30 mg. Ca in form of $CaCl_2$ was measured into a number of tubes, and varying amounts of 0·1 *N* NaOH solution added to each. Methyl red or phenol red was added to the tubes, which were then corked and kept at 37° during five days with frequent shaking. At the end of this time the p_H of the supernatant liquid in each tube was noted and was as follows:

cc. 0·1 *N* NaOH	p_H	
3·0	4·5	3·23 cc. *N*/10 NaOH required to form $R^1_2HPO_4$
6·3	5·0	
6·5	5·2	
6·8	5·4	6·46 cc. *N*/10 NaOH required to form $R^1_3PO_4$
7·0	5·8	
7·3	6·8	
7·6	7·8	

In absence of calcium the addition of 3·23 cc. 0·1 *N* NaOH would have raised the p_H to about 9·0 and to about the same value had a portion of the phosphate been precipitated as $CaHPO_4$. But double the above amount of NaOH was added without raising the p_H above 5·2 which proves that at this p_H the solid deposited must have been tertiary calcium phosphate or a still more basic compound. By the sudden addition of a large excess of calcium or phosphate, $CaHPO_4$ may of course be thrown down, but the solution will not be in true equilibrium with the solid phase, and some of the $CaHPO_4$ must re-dissolve and $Ca_3(PO_4)_2$ be precipitated until the p_H is thereby lowered below the limiting value (probably below 5·0).

For the sake of greater lucidity in the above paragraphs, we have considered HPO_4 and PO_4 ions alone, but the argument applies equally to other ions such as those derived from the still more basic hydroxyapatite, if, as Bassett suggests, this, and not the tertiary phosphate, is the compound present in bone.

Summary.

The phosphoric esterase discovered in bone and ossifying cartilage is also present in equally high degree in the teeth, especially those of very young animals.

The optimum p_H for this enzyme lies on a flat curve between 8·4 and 9·4, a reaction which is in other ways favourable for the precipitation of tertiary calcium phosphate and calcium carbonate.

The bone enzyme also hydrolyses ethyl butyrate and triacetin, but has no action on olive oil. The enzyme is however distinct from the esterase of pancreas (and probably also those of the liver and spleen) which is relatively inactive towards phosphoric esters.

Deposition of fresh calcium phosphate has been demonstrated in bones taken from rachitic rats and immersed in solutions of calcium hexosemonophosphate or calcium glycerophosphate at 37° and p_H 8·4–9·4.

These experiments further support the belief that the enzyme is actively concerned in ossification and also furnish evidence that the enzyme is secreted in the region of the osteoblasts and hypertrophic cartilage cells.

The high optimum p_H of the enzyme further suggests the possibility that the reaction of the tissue fluids in this region may by some mechanism be rendered more alkaline than that of the blood, thereby increasing the activity of the enzyme and providing favourable conditions for the deposition of calcium salts.

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